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Procedia Environmental Sciences 23 (2015) 329 – 337

Procedia Environmental Sciences

International Conference on Tropical and Coastal Region Eco-Development 2014 (ICTCRED 2014)

Detection of Bacteria and Fungi Associated with *Penaeus monodon* Postlarvae Mortality

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Abstract

Diseases on shrimp postlarvae caused by bacterial and fungal still remain an important concern due to their economic values. Application of microalgae as natural feed has brought promising results for growth of shrimp postlarvae. The aim of this research is to apply fusant of *Dunaliella* and *Chlorella* microalgae and to detect feed effect on microbial diseases resistance on postlarvae. The research was conducted using bacterial and fungi from contaminated shrimp postlarvae. Microbial species were characterized based on morphological and biochemical tests. Total number of bacteria ranging from 21.7×10^5 cfu to 32×10^5 cfu while fungal calculation about 6×10^2 cfu. Microorganisms analysis presumably belong to genus *Vibrio, Pseudomonas, Aeromonas, Alcaligenes, Bacillus, Staphylococcus, Hafnia* and *Fusarium*. The absence of *V. harveyi* pathogen indicated that the fusant serve as the main source on increasing of resistance to diseases and thus reducing the mortality of shrimp postlarvae.

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Peer-review under responsibility of scientific committee of the ICTCRED 2014

Keywords: postlarvae, *Penaeus monodon*, bacteria, fungi, mortality

Introduction

Tiger shrimp (*Penaeus monodon*) was one of the leading aquaculture commodities in Indonesia which have been

Peer-review under responsibility of scientific committee of the ICTCRED 2014
doi:10.1016/j.proenv.2015.01.048

developed rapidly. However, this promising potential have to faced serious problems attributed to bacterial and fungal diseases causing mortality of shrimp postlarvae[1]. Postlarvae evaluation is the key in shrimp diseases control[2]. Diseases caused mainly by bacterial followed by fungal infections [3].

Supplementation feeding on *Penaeus monodon* postlarvae with microalgae exhibits significant effect on growth, weight, survival and immune response in high and low salinity. Carotenogenic microalgae which live in different environmental salinity, namely *Chlorella* and *Dunaliella* are needed for survival of shrimp postlarvae during their life cycle[4][5]. Their application as natural supplement was potential to subtitute synthetic feed since it contains proteins, carbohydrates, lipids and vitamins, carotenoid as antioxidants, and trace elements[6][7].

Improvement of valuable metabolites from *Chlorella* and *Dunaliella* microalgae was done using biotechnological methods employing somatic hybridization by protoplast fusion[8]. This intergeneric fusion enables nuclear and cytoplasmic genomes to be combined, fully or partially, at the intergeneric levels to circumvent naturally occurring sexual incompatibility barriers[9][10]. Fusant microalgae has gained considerable importance in combining valuable product on both microalgae. It also offers more promising positive effect as feed supplement for shrimp postlarvae. This methods are applicable widely such as in the production of biofuel[11], biomass, pharmaceutical, etc. [9]. Relatively litle information is available in the literatures regarding the performance of shrimp cultured on a diet supplemented with fusant product from *Chlorella* and *Dunaliella* microalgae related with microbial diseases resistance. In this paper, a possibility of fusant supplement used to reduce microorganisms contamination related with mortality and diseases resistance of *Penaeus monodon* postlarvae was studied.

2. Materials and methods

2.1. P. monodon

Postlarvae *P. monodon* were obtained from Brackishwater Aquaculture Development Centre (BBPBAP) on Jepara Indonesia. They were held in seawater tanks, recirculated and aerated, with the temperature set at 25-28^{o}C and salinity at 30-32‰. The tanks were cleaned daily. The shrimps were fed with pellet or synthetic feed as a control. The stage of shrimp were chosen at the postlarvae stage for 10-12 days (P.L.10-P.L.12), which making the shrimp capable of withstanding transportation, acclimation and stocking at the farm due to complete gill development

2.2. Microbial media

The nutrient agar media (NA) (Merck), Nutrient Broth (NB) (Merck) and Potatoes Dextrose Agar (PDA) (Merck) media were prepared by dissolving them in distilled water and was autoclaved.

2.3. Isolation of microorganisms

The samples of affected *P. monodon* were collected and homogenized and further used for bacterial and fungal isolation[2][11]. Then, the homogenated sample was serially diluted from 10^{-1} to 10^{-6}. And, 0.1 ml of sample was taken from 10^{-4} , 10^{-5}, and 10^{-6} diluted sample, followed by spreading over them on NA and NB medium separately for bacteria, and the PDA for fungi. One media from each of them was maintained as a control without sample. The plates were incubated at 37^{o}C for 24 to 48 hours and the bacterial colonies was calculated using TPC Method.

2.4. Identification of shrimp bacteria

The isolated bacterial species were identified based on the morphological and biochemical characteristics of the individual colony and recorded. The individual colony of bacteria was transferred to NA and NB. The isolates were

subjected to different morphological and biochemical test include Gram staining, motility test, gelatin liquefaction, casein hydrolysis test, catalase test, nitrate test and carbohydrate fermentation test, growth on salinity test and colony pigment appearance[13] [14] [15].

2.5. Identification of shrimp fungi

The isolated species of fungi were identified based on morphological characteristics of the individual colony and cell. The fungi were cultured into PDA media and incubated at 25-28^{o}C for 1-7 days. The different morphology of fungi was transferred to PDA agar. The fungi identification was performed according to the usual morphological criteria[12]. Observation and identification of the fungi cell were performed after 10 days of culture with lactophenol and observed microscopically.

2.6. Diet with feed of fusant microalgae

Estimation of supplement fusan feed needed by one shrimp was calculated based on microalgae levels in shrimp gastrointestinal tract (GIT). Fusant of *Chlorella* and *Dunaliella*, was administered at 8-10^4 cells each day for 1 feed to shrimp postlarvae during eight weeks based on their final weight. Comparison was made for untreated control group fed by synthetic pellet. Each postlarvae was having the same weight gain. Significant differences for specific growth rate (SGR) and survival were recorded in shrimp fed diet as compared with the control as supported data. Eight weeks after the feeding period, death shrimp from both treatments were followed with identification of microbial contamination.

Results and Discussion

3.1. Bacterial Identification

Post-larval stages of the life cycle is the most vulnerable to shrimp disease. The present study deals with the distribution of bacteria and fungi which suspected to be the major reason in causing mortality of *P. monodon* postlarvae from hatchery. Of almost 2000 samples of shrimp postlarvae, the total number of isolated bacteria were 21.7×10^6 from shrimp postlarvae with treatment, and 32×10^6 CFU/g of from larval macerate of control. While total number of fungi from both treatments was almost the same, which is about 4×10^2. This bacterial number was very important to notes since one of the first criteria of microbiological test for evaluating shrimp postlarvae quality is stated that maximum total bacteria count of 1.0×10^3 CFU/g of larval macerate in agar, of which more than 90% of the colonies should be yellow[2].

The research result show that the occurence of total bacteria in *P. monodon* postlarvae exceeded the allowed maximum number. This result indicated that mortality of postlarvae was mainly caused by bacteria. Furthermore, most of the colony on NA plate had a yellow colour, instead of white and pale. This may be possibly due to presence of suspected several genus *Vibrio* and its related genera consist of *Aeromonas, Hafnia, Pseudomonas* and *Alcaligenes.* From the result we have to be aware because early studies suggested that, in recent years, some *Vibrio* strain are pathogenic and can cause Vibriosis, a serious infectius disease in maricultured organisms[16]. Several Vibrios associated with shrimp postlarvae, juvenile and adult stages consist of *V. alginilyticus, V. parahaemolyticus, Photobacterium damselae,* and *V. mimicus*[17].

Identification based on the morphological and biochemical characteristics compared with Bergey's manual of determinative bacteriology is tabulated on Table 1. Based on the comparison, the bacterias were confirmed as a member of genus *Bacillus,Vibrio, Aeromonas, Alcaligenes, Hafnia, Pseudomonas, and Staphylococcus.* Another scientist supported this finding that disease in black tiger shrimp postlarvae is caused by bacteria of the genus *Aeromonas, Pseudomonas*, and *Plaubacterium*[1][18]. Slightly different result was getting *Aeromicrobium*

erythreum, B. subtilis, Escherichia coli, V. cholerae, Enterobacter aerogens, P. putida, P. aeruginosa, Brucella canis and *Enterococcus pseudo avium*[19].

Table 1. Characteristics of the genus of selected bacteria

Character	Bacterial Colonies									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Pigment colony	w	y	y	y	y	y	y	y	y	y
Gram	+	-	-	+	-	-	-	-	-	+
Luminescence	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	-	+	+
Casein hydrolysis	+	+	+	+	+	+	-	+	+	+
Gelatin liquefaction	-	-	-	-	+	-	-	-	-	-
Carbohydrate metabolism (O/F medium)	f	f	f	f	f	f	f	f	-	f
Gas production	+	-	-	+	+	-	+	+	-	+
Acid production	-	-	-	+	-	-	-	+	-	-
Growth at 5° C	-	-	-	-	-	-	-	-	-	-
Growth at 37° C	+	+	+	+	+	+	+	+	+	+
Growth at 42° C	-	-	-	+	-	-	-	-	-	-
Growth without added NaCl	+	+	+	+	+	+	+	+	+	+
Growth in 3.0 % NaCl	+	+	+	+	+	+	+	+	+	+
Growth in 4.0 % NaCl	+	+	+	+	+	+	+	+	+	+
Growth in 6.0 % NaCl	+	+	+	+	+	+	+	+	+	+
Growth in 7.0 % NaCl	+	+	+	-	+	+	-	+	+	+
Growth in 7.5 % NaCl	+	+	+	-	+	+	-	-	+	+
Growth in 8.0 % NaCl	+	+	+	-	+	+	-	-	+	+
Growth in 10.0 % NaCl	+	+	+	-	+	+	-	-	+	+
Growth in 15.0 % NaCl	-	-	-	-	-	-	-	-	-	-
Identified of bacterial genus	*Bacillus*	*Vibrio*	*Vibrio*	*Bacillus*	*Aeromonas*	*Vibrio*	*Alcaligenes*	*Hafnia*	*Pseudomonas*	*Staphylococcus*

w= white, y = yellow, + = positive, - = negative, f= fermentative

The distribution of bacteria on Fig. 1. also suported the result in showing widespread distribution of the common Gram negative bacteria in the outer area of shrimp postlarvae comparing with inner area of hepatopancreas. This result indicated that Gram negative bacteria mainly came from coastal, fresh or seawater as their commonly habitat.

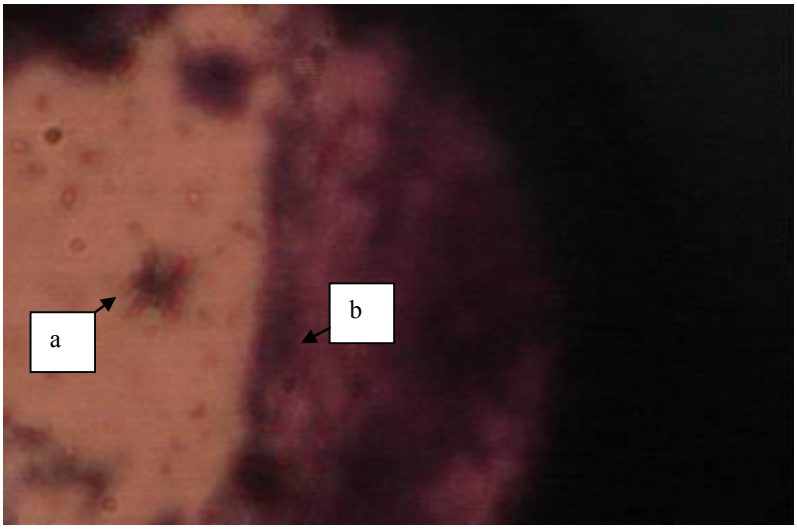

Fig. 1. Distribution of Gram negative (a) and Gram positive (b) bacteria in hepatopancreas area of shrimp postlarvae

However, despite the above result, second evaluation for good quality of shrimp postlarvae was depend on the presence of *V. harveyi* (bioluminescent bacteria), which can be detected in agar. In marine invertebrates, notably larval penaeid shrimp, *V. harveyi* has become a major constraint on production, particularly in Asia[19][20]. The result shows that from ten selected bacteria isolates on luminescent test medium (Table.1 and Fig. 2), luminescent bacteria like *V. harveyii* from the samples did not exist. It was suggested that *V. harveyi* is a marine Gram-negative luminous organism with a growth requirement for sodium chloride[21][22]. Further observation using 3.0-15.0 % of NaCl (Table 1) did not show luminescence area as an indicator of *V. harveyi* existence. Luminous bacterial disease in Indonesia occur during the rainy season which decrease salinities (10-15 ppt) and bases pH resulted in significantly enhanced penaeid prawn postlarvae mortalities[24].

The result exhibited negative incidence of luminescent bacteria during postlarvae stage in the rearing tank water could be an indicator that the environment still having good quality. These result having implication that the early larval stages and the research environment was not giving possibility to development of luminuous bacterial disease and presence of bacteriophages in the larval rearing tanks. The filtered intake sea water was providing some advantageous effect in disminishing the load of *V. harveyi* in the hatchery[23].

The occurence of Gram negative bacteria was more frequent comparing with Gram positive ones in this research which can be seen in Fig. 1 and Fig. 2. The genus of *Vibrio* was belong to the same Family of Vibrionaceae together with *Aeromonas* and *Pseudomonas*. They also having close relationship with some other genus *Hafnia,* and *Alcaligenes* as the same Gram negative and rods bacteria.

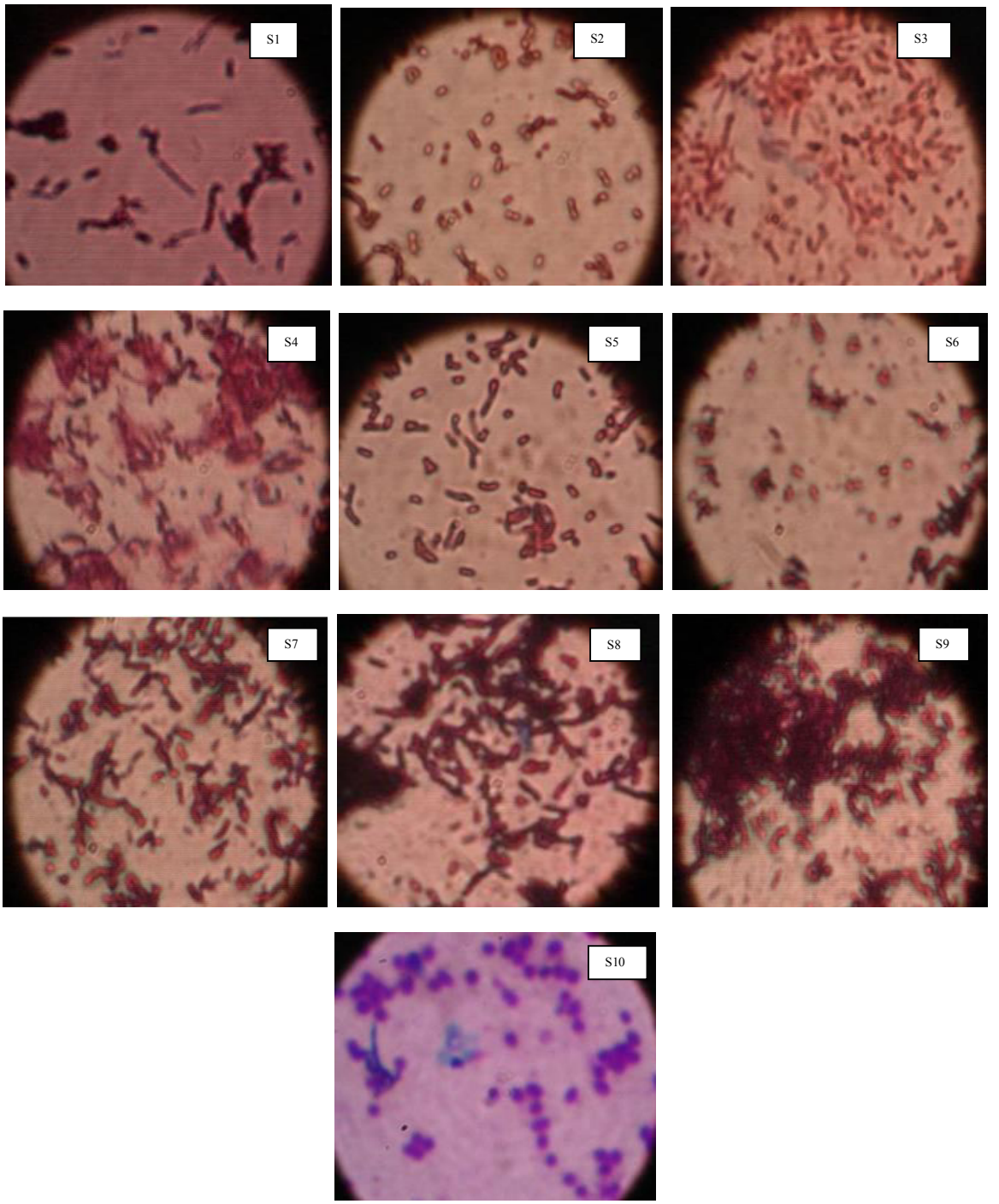

Fig. 2. Microscopic appearance of selected bacteria (S1-S10) in death postpostlarvae of *P. monodon* after 24 hour incubation (1000 ×)

3.2. Fungal Tests

Fungal identification according to the usual morphological criteria showing existence of *Fusarium* genus based on its phialide, microconidia and macroconidia. The colony was growing fast with pale colour (whitish to cream). Aerial mycelium was absent. Conidiophores of *Fusarium* usually basitonously branched. In some species reduced to single phialides, also sometimes forming complex pustules (sporodochia) or forming a confluent slimy mass of spores with a fatty or greasy appearance[12]. Two type of conidia can be distinguised into macroconidia and microconidia. However, detailed identification must be done by inoculated colonies and incubated in diffuse daylight at 20-25^{o}C for 10-14 days. Identification of the species can be very difficult because of the variability between isolates and because not all the features develop fully.

The fungi on *P. monodon* postlarvae was observed in almost surface of the shrimp body. This result also in agreement with other result which suggested that fungi in penaeid shrimps live in the body of the shrimp postlarvae and subsist on the body tissues as its live media[1]. The research was done during February untill May that can be considered as dry season in Indonesia which favors fungal growth. The diseases on shrimp usually caused by fungi from genus of *Fusarium*. Other researcher stated that a gill-blackening disease in *P. japonicus* caused by parasite *F. oxysporum* will decrease their hypo-osmoregulatory and hyper-osmoregulatory[25]. The pathogenic effect of *F. oxysporum* is dose- and isolate-dependent. Moulting or exposure to low salinities increased animal mortality. This pathogen is able to produce *in vitro* macromolecular toxic compounds disturbing the osmoregulation of shrimps. Fusariosis and black gill disease caused by *Fusarium* spp may affect all developmental stages of Penaeid shrimp. *Fusarium* spp are opportunistic pathogens that may lead to high mortalities (90%). Disease is noticed in ponds where water quality management is poor[26].

The observations made in the present study showed that despite the potency of bacteria and fungi that can act as pathogen caused shrimp larva mortality, microbial identification result also showed the potency of these microorganisms to support shrimp larva survival. Recent studies showed that genus *Bacillus* as one of genera found in *P. monodon* postlarvae, having potential use as probiotic feed supplement that increased immunity to *V. harveyii*, including a reduced mortality[27][28][29][30]. Furthermore, some other bacteria consists of several strain *Bacillus, B. subtilis, B. cereus, V. pelagius, V. mediterranei, A. media, Pseudomonas* and *Thalassobacter utilis* also can be used as probiotics or biological control againts *Vibrio* also in turn as a potent growth promoter, immuno enhancer, improve the health and survival for *P. monodon* postlarvae. This report is substantiated in this research, by a positive correlation between the negative existence of *V. harveyi* in the death postlarvae of *P. monodon*. *B. pumilus* naturally constitute a part of the bacterial flora of the shrimp intestinal tracts. This non-pathogenic probiotic bacteria having high specificity to the cultured shrimp host and provide a healthy balance of indigenous organisms in the hosts intestines.

It has been reported that microalgae *Chlorella* sp. serve as immunostimulant for shrimp postlarvae[31]. *Chlorella* is considered as the major source of lutein and astaxanthin to be used as feed supplement for shrimp postlarvae and antioxidants[32][33]. Another important substance in *Chlorella* sp. is beta-1,3-glucan which considered responsible to initiate host defense reactions in response to pathogen surface molecules. Protoplast fusion recombinant will contain valuable nutrition from *Chlorella* sp. in combination with large numbers of β-carotene from *Dunaliella*[34][35]. This report is showed positive correlation between the survival of shrimp postlarvae and application of the fusant as feed supplement. It was predicted strongly that application of fusant as the major reason for supporting the degree of fitness of shrimp postlarvae and, decreasing mortality and reducing contamination of microorganisms including *V. harveyi.*

4. Conclusion

The study has showed that application fusant of *Dunaliella* and *Chlorella* microalgae as natural feed on tiger shrimp postlarvae has decreased contamination of bacteria and fungi. The implication of the research exhibited the potential of fusant in increasing the resistence to microbial diseases and reducing shrimp postlarvae mortality. The study has

also indicated that the possibility of microorganisms on shrimp postlarvae as probiotic which remain to be explored.

Acknowledgments

This research was funded by Directorate General of Higher Education (DP2M-DIKTI), Indonesian Ministry of Education and Culture, with the contract number 023.04.02.189185/2014.

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